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Use of Fluorochromes for Direct Enumeration of Total Bacteria in Environmental Samples: Past and Present

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INTRODUCTION

Quantification of bacterial numbers and biomass is important to our understanding of the ecological role of bacteria in any environment. Identifying the primary factors responsible for the regulation of bacterial numbers is a major goal of microbial ecology, hence the need for an assessment of methods. Tracing historical patterns in the use of these methods provides a greater understanding of the current status of chosen techniques.

Bacteria have been enumerated in several ways, with many of the same techniques being applied in diverse systems. Increasingly, direct counts are used in studies of bacterial population densities rather than traditional spread plate methods, which have been shown to significantly underestimate numbers (24, 97). Epifluorescent direct count techniques are frequently the methods of choice, yielding more accurate estimates of total (including nonviable and viable but nonculturable) cell numbers in a wide variety of situations. Routinely ignored has been the fact that differences in numbers of bacteria observed depend on the staining technique and physicochemical characteristics of the samples (12, 33, 50, 60, 64, 75, 85), as well as individual investigator bias. Precautions must be taken if accurate and reproducible quantification is desired.

In this review we summarize our results obtained during an examination of over 220 papers describing studies in which fluorochrome staining followed by epifluorescent microscopic direct counts was used to estimate total bacterial abundances. A complete bibliography of reviewed papers is available (hard copy or diskette) from the authors upon request.

ACRIDINE ORANGE AND DAPI

Utility of Acridine Orange and DAPI Staining

Epifluorescent direct counting is the best method available for the enumeration of total bacteria in environmental samples (15, 24, 32, 39). The two fluorochromes most often used in direct count methods are 3,6-bis[dimethylamino]acridinium chloride (acridine orange [AO]) and 4′,6-diamidino-2-phenylindole (DAPI). With both stains, bacteria are identified on the basis not only of color but also of size and shape. Distinguishing cells on the basis of morphology is important because no fluorochrome is truly specific to bacteria. AO binds to both DNA and RNA with an excitation maximum of approximately 470 nm. AO-stained single-stranded nucleic acids emit orange-red fluorescence, while those that are double-stranded tend to fluoresce green in vivo (49, 69). It is thought that AO binds primarily to adjacent phosphate groups in the nucleic acid backbone and that orange fluorescence is due to dye-dye interactions that require high dye/nucleotide ratios (5, 17). The distribution of dead, metabolically inactive but living (moribund), and living cells cannot be determined by the standard technique of either AO or DAPI staining, because DNA retains its staining properties even in nonviable cells. Although both DAPI and AO stain bacteria and other fine particulate organic matter differentially, little can be said concerning the physiological state of bacterial cells on the basis of color differentiation outside of well-defined laboratory conditions (57).

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Since its introduction (19, 64), the fluorochrome DAPI has been rapidly replacing AO as the most commonly employed bacterial stain for a wide range of sample types. DAPI is a nonintercalating, DNA-specific stain which fluoresces blue or bluish-white (at or above 390 nm) when bound to DNA and excited with light at a wavelength of 365 nm. When unbound, or bound to non-DNA material, it may fluoresce over a range of yellow colors. Although DAPI may stain polyphosphate (1), preferred binding sites are A+T-rich DNA sequences, with a minimum binding requirement of at least three consecutive A·T pairs. Other, less frequently used, stains include acriflavine, bisbenzimide (and other Hoechst dyes), erythrosine, fluorescein isothiocyanate, fluorescamine, rhodamine, rose bengal, euchrysine-2GNX (3,6-diamino-2,7-dimethyl-9-methylacridinium chloride; also an acridine derivative), ethidium bromide, berberine sulfate, phenolic alanine blue, methylene blue, and several others, none of which are considered in this review. Recently, the use of fluorescent DNA probes or fluorochrome-labeled antibodies in combination with a general fluorochrome (e.g., DAPI) has permitted estimates of the proportion of total bacterial cells within specific serotypes or taxa. In this paper, we document the increasing use of DAPI by environmental microbiologists to enumerate total bacteria, provide information on additional trends in the application of DAPI and AO for the enumeration of bacteria in a variety of sample types, and present a technical framework which is based on consideration of previous methodological studies.

Sample Types

Microbiologists are often interested in determining and understanding organism abundances in particular habitats or sample types. The earliest uses of epifluorescence microscopy encountered involve the pioneering work of Strugger and his enumerations of bacteria in soil suspensions. He used "acridinorange (3,6-tetramethyl-diaminoacridin)" throughout the 1940s and can be credited with publicizing one of the primary advantages of AO over previously used stains (see reference 84 and references therein), namely, the ability to distinguish bacterial cells from interfering soil particles on the basis of color. Direct microscopic techniques for enumerating soil (see, e.g., references 20, 21, and 93) and aquatic (see, e.g., reference 68) bacteria pre-date Strugger's work, but it was only with the advent of fluorescence microscopy that researchers discovered bacterial abundances exceeding spread and pour plate method estimates by 10 to 10^4 times (24, 27, 42, 53, 82, 83). Direct microscopic enumeration has shown that numbers of bacteria capable of forming colonies on "nonselective" media are usually several orders of magnitude fewer than numbers actually present and metabolically active in freshwater, marine, and soil environments (see reference 72 and references therein). Thus, improved methods have given the role of heterotrophic bacteria new significance in both terrestrial and aquatic systems.

Epifluorescent direct count procedures involving AO or DAPI have been used on a diverse collection of samples, ranging from Antarctic soils (13) to oyster tissue homogenates (22) (Table 1). Most work has been on field samples returned to the laboratory for bacterial enumeration. Reviews of the literature in other specific disciplines (e.g., environmental engineering, medicine, and food sciences) would significantly increase the listing of specific sample types to which these procedures have been applied.

The majority of reviewed publications involved examination of samples from aquatic environments (Fig. 1). AO, in use for more than 50 years, has been applied to a wider range of sample types than DAPI. The standard AO technique now used (31, 40) was originally developed for seawater samples. DAPI has been used mainly with saltwater or freshwater samples, with the majority of papers dealing with bacterial numbers in lakes, ponds, and lotic habitats. Both DAPI and AO have been used extensively with aquatic sediments, and a recent review of factors controlling bacterial numbers and production in marine and freshwater sediments cites bacterial abundance data from 26 studies, 25 of which used either DAPI or AO staining to obtain results (74). Of the papers examined in the present review, relatively few have described studies in which DAPI methods have been used with natural, nonaqueous samples, although this may reflect a bias in the type of literature reviewed.

TABLE 1. Types of samples to which AO and DAPI staining methods have been applied for the direct enumeration of bacteria

Freshwater	Surfaces
Lake water	Epiphytic bacteria (direct)
Pond, bog, and river water	Epiphyte suspensions
River and reservoir seston	Epixylic suspensions
Tree-hole (phytotelmata) water	Macrophyte detritus
Municipal wastewater, sewage	Deciduous leaf discs
Activated sludge	Colonized litter bags
Stream water	Stream periphyton
Spring water	Epizoic bacteria
Artificial pond water	Membrane filters
Mixed and pure bacterial cultures	Glass slides
Freshwater swamp cultures	Biofilms on stainless steel
Groundwater	
"Freshwater samples"	
Saltwater	Other
Seawater	Milk, apple juice, other beverages
Estuarine water	Various food suspensions (whole peppers, meat, donuts, etc.)
Seawater microcosms	Intravenous fluids (NaCl and glucose solutions)
Seawater batch cultures	River macrophyte leachate
Estaurine cultures	Fecal pellets
Bacterial/diatom cultures	Crab pseudofeces
Tidal creek cultures	Homogenized worms
	Homogenized molluscs
Soil/sediment	
Soil suspensions	
Soil smears	
Marine sand	
Salt marsh sediment	
Marine intertidal sediment	
Marine epilithon	
Lake sediment	
Stream sediment	
Stream epilithon	
Deep subsurface sediment	
Mangrove swamp sediment	
Saline pond sediment	

Historical Development

Developments in epifluorescent bacterial direct counts between the 1940s and 1970s mainly involved improved cell-staining procedures (45). At that time, the general consensus was that acridine-based fluorochromes yielded the best estimates of cell numbers. Work by Strugger in the 1940s (with soil bacteria) and by Jannasch in the 1950s and 1960s (with planktonic bacteria) showed AO to be an effective bacterial stain. For soil bacteria, some researchers found fluorescein isothiocyanate to yield higher counts than AO (4) while others

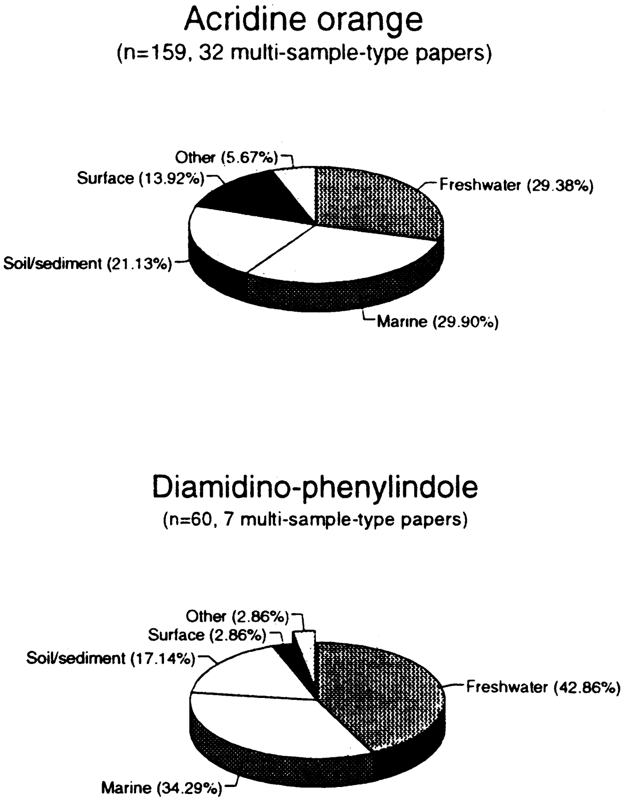

FIG. 1. Percentage of samples by type (see Table 1) in which bacteria were enumerated following staining with AO or DAPI (*n* = number of publications).

observed the opposite (56). Other studies in the 1970s compared the effectiveness of AO with that of other stains (25, 43, 45, 96), and throughout this time AO was generally accepted as the best bacterial stain.

A major breakthrough in the use of epifluorescent counts was the first use of polycarbonate Nuclepore membrane filters, prestained with irgalan black (IB) to reduce background autofluorescence (40). The idea of counting bacteria directly, following concentration on membrane filters, dates from much earlier (see reference 29 and references therein). However, bacterial cells became embedded and impossible to count in the rough surfaces of the cellulose filters traditionally used, and Hobbie et al. (40) obtained counts twice as high by using the smoother Nuclepore filters. Although Nuclepore filters had been used earlier (96), destaining and rendering filters transparent to reduce background fluorescence made this method very time-consuming. Of 144 papers published since 1977 describing studies in which AO was used, 91 cite Hobbie et al. (40) in some context and 67 cite this seminal work as the sole reference for their bacterial count method, even though Hobbie et al. (40) recognize Francisco et al. (31) as the originators of the basic method. Interestingly, Francisco et al. (31) cite Hobbie (in the form of a personal communication) regarding the use of epifluorescence microscopy for the examination of stained membrane filters.

Ramsay (66) improved on the technique of Zimmerman and Meyer-Reil (96), in which the bacteria are stained after being filtered instead of in solution (the "filter-stain" method as opposed to the stain-filter method). She found that bacterial densities were much less variable than actual heterotrophic activity, expressed on a per cell basis, in both freshwater and marine samples. Previously, aquatic samples had been enumerated by the filter-stain method (10, 31). A degree of success was also realized in the application of previous AO staining techniques to an expanding variety of sample types, such as epiphytic bacteria (34, 59, 67, 73).

Sixty-four papers describing studies in which DAPI was used to stain bacteria were reviewed. Porter and Feig (65) are cited in 53 of these papers and are cited as the sole reference for the chosen method in 37. Additionally, Hobbie et al. (40) are cited in 18 of these 64 publications (including the paper by Porter and Feig [65]), usually regarding the use of prestained polycarbonate membrane filters. Appearing in the same issue of *Limnology and Oceanography* as the oft-cited work of Porter and Feig, is another of the earliest papers describing the use of DAPI is for counting total bacteria (19). In this paper Coleman also introduces the virtues of DAPI staining, yet this work is rarely cited (3 of our 64 DAPI papers). The convention since 1980 has been to cite Hobbie et al. (40) when AO is used and both Porter and Feig (65) and Hobbie et al. (40) when DAPI is used. As examples, the literatures on AO and DAPI staining show that the evolution of accepted methods are not likely to be straightforward or attributable to a single lineage.

We estimate that between the 1940s and 1980, roughly 90% of all bacterial direct counts were performed following staining with AO. Jones (43) reported that AO and fluorescein isothiocyanate were the two fluorochromes in most-common use. Within the last decade however, DAPI has largely displaced all other non-acridine-based fluorochromes (including fluorescein isothiocyanate). Of studies involving only AO or DAPI, 50% have used DAPI since 1980, whereas over 70% have used DAPI since 1988 (Fig. 2). These figures indicate that DAPI is rapidly replacing AO as the bacterial stain of choice.

METHODS FOR DIRECT ENUMERATION

Preservation

Samples for bacterial enumeration should be preserved immediately following collection to avoid changes in numbers, sizes, and shapes of bacteria which may occur rapidly (often in less than 1 day) with storage (32). Of 150 publications randomly reviewed for information on preservation of samples for bacterial direct counts by epifluorescence microscopy with either DAPI or AO, 45 contained absolutely no information on sample preservation or fixation. Of those that did, the vast majority reported preservation with aldehyde solutions.

The most commonly used preservative is formaldehyde (FMA), which acts to harden bacterial cells, thus preventing damage during homogenization or sonication (33). Aldehydes function by cross-linking proteins in cell membranes, thus improving bacterial cell rigidity (17). FMA-based preservatives were used in the studies described in 88 of the 105 papers mentioning sample preservation. Of these, 46 reported the use of formalin while 41 reported the use of FMA; the distinction between formalin and FMA is quite important. FMA is a gas at temperatures above −21°C, while formalin is the commercially available liquid form of FMA. Formalin is usually a 36.8, 38, or 40% (vol/vol) aqueous solution of FMA. Thus, a 2% FMA solution is not the same as a 2% formalin solution. Unless concentrations are specified in terms of actual FMA content, dilutions are usually considered in terms of the commercially available product; e.g., a 10% solution would be 1 volume of concentrated formalin (40% FMA-saturated water) to 9 volumes of water (41). FMA is also available as a solid polymer, paraformaldehyde, but this has been infre-

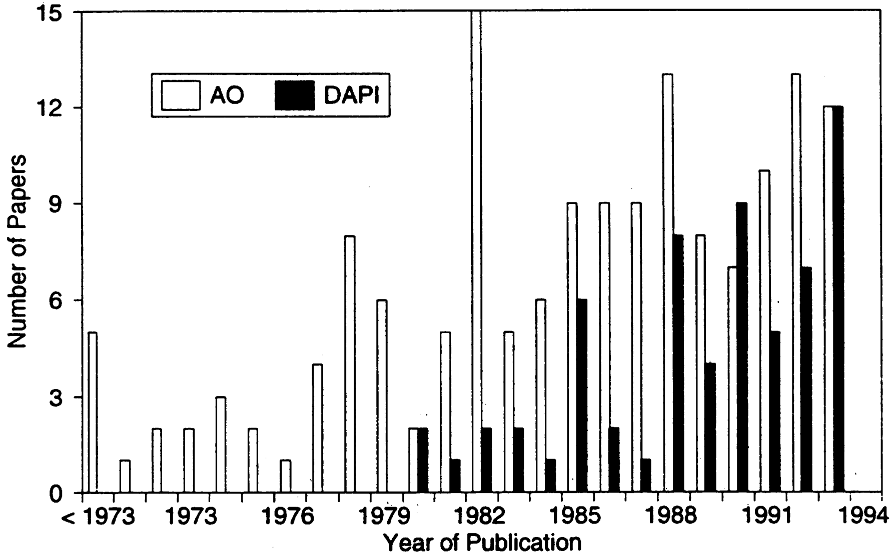

FIG. 2. Number of reviewed publications over time in which either AO (n = 159) or DAPI (n = 60) was used as a bacterial stain for epifluorescent direct counts.

quently used for preserving samples. Glutaraldehyde (GTA) use was reported in 20 of 105 papers, and only 3 papers reported the use of nonaldehyde preservatives. Four papers reported the use of more than one type of preservative.

A summary of preservative concentrations, assuming that the formalin used in all studies was a 40% (vol/vol) FMA solution, is provided in Table 2. Formaldehyde was used most often at a final concentration of 2%, although concentrations ranging from 0.005 to 10% were reported. The quantity of irreversibly FMA-bound protein drops as the pH of the solution rises above 10; thus, formalin reacts most efficiently as a solution buffered near neutrality (pH 7.5 to 8.0 [41]). Preservatives are often phosphate, acetate, borate, or NaCl buffered, and alkaline buffering of preservatives is recommended to avoid nucleic acid degradation and cell lysis resulting from the decomposition of aldehydes to their corresponding acids in the presence of molecular oxygen (24). The use of buffered aldehyde preservatives was described in 20 papers of the 150-paper subset.

In some cases, use of FMA as a preservative is contraindicated. Chlorophyll pigment autofluorescence may fade within 24 h following preservation in 1% FMA. Therefore, preservation in 1% GTA is recommended if differentiation between phototrophic (i.e., chlorophyll-bearing) and heterotrophic microorganisms is desired (28). GTA is most frequently used at a concentration of 1%, although Clarke and Joint (18) found no change in cell numbers for up to 1 month with storage in 2.5% (vol/vol) electron microscope-grade GTA. Alternative preservatives are recommended for nonbacterial microorganisms; these include van der Veer fixative (90), Lugol-formalin-thiosulfate mixture, 4% ice-cold GTA (78, 79), GTA-osmium tetroxide fixative (80), and GTA-paraformaldehyde combinations (48). All preservatives should be filter sterilized (pore size, 0.2 μm) prior to usage to avoid sample contamination.

TABLE 2. Reported concentrations of GTA and FMA used to preserve samples for bacterial enumeration in 150 studies

Preservative	Mean[a]	Mode[a]	SD[a,b]	Range[a]
GTA	1.78	1.0	1.50	0.1–5.0 (n = 19)
FMA	1.87	2.0	1.69	0.1–10.0 (n = 82)

[a] All values are percentages (vol/vol).

[b] SD, standard deviation.

Although soil and sediment samples are sometimes frozen, all sample types are normally preserved chemically. These preserved samples are best kept refrigerated (4 or 5°C) in the dark. Fry (32) reports the successful preservation of seawater and marine sediment in 0.2% FMA for up to 10 days with no significant change in bacterial numbers or biovolumes. Water samples stored at 5°C in 2% FMA yielded consistent AO counts for 1 to 2 weeks (25). One source indicates that water samples may be stored for up to 3 weeks without a significant decrease in cell numbers following fixation in 5.0% (wt/vol) phosphate-buffered GTA and storage at 4°C in the dark (2). However, decreases in AO counts have been noted within 40 days of storage in GTA, and decreases in DAPI counts of formalin-fixed samples stored under refrigeration have been observed (89). Once slides are prepared, the longevity of the samples may be dependent on which fluorochrome was used. Counts of AO-stained samples decrease within 1 week regardless of storage temperature, whereas DAPI-stained slides yield consistent counts for up to 24 weeks when refrigerated (4°C) in darkness (65). Prepared slides of seawater samples may be stored frozen (−20°C) for up to 70 days with no appreciable loss in cell numbers (88). Given the paucity of information regarding the effects of long-term storage on other sample types, we recommend that slide preparation and counting take place as soon as possible following sample collection.

Dispersion

Recent work has dealt with the examination of samples bearing sediments, or other nonliving particles, which may interfere with the visualization of microorganisms. Prior to the advent of particle dispersion and filtering techniques, soil-agar smears were prepared on microscope slides, stained, rinsed, and observed directly (87). Infrequently, some soil microbiologists may stain and count bacteria that have colonized glass slides placed in the field (see, e.g., reference 61) rather than collect soil samples. More typically, techniques are used to disperse particle-attached bacteria in soil or sediment slurries. Bacteria attached to surfaces may be removed by standard procedures (38), and samples may be stained as a suspension for direct counting following filtration. Dispersion and disaggregation are important because accurate counts can only be obtained if cells are distributed evenly on filters.

In this case, bacterial staining becomes the second part of a two-stage process first involving the separation of microorganisms from mineral particles and detritus. Blending, stomaching, ultrasonic cavitation, or other homogenizing treatments of samples are often combined with additions of chemical dispersants such as the surfactants Tween 80 (95), Na_4PP_i (91, 92), or Triton X-100 (54). Bakken (9) tested several detergents and buffer solutions as bacterial dispersants, including sodium hexametaphosphate buffered with Na_2CO_3 (Calgon), Tween 80, Na_4PP_i, Winogradsky's salt solution, and bromhexinchloride (Bisolvon). For recovery of viable bacteria, Scheraga et al. (76) recommend shaking of samples in the presence of 0.00001% cetyltrimethylammonium bromide.

Various mechanisms involving physically agitating samples to aid dispersion have been tested and compared (9, 30, 54, 91, 95), and different methods have been recommended for various sample types. Mechanical homogenization may be the best way of removing bacteria from sediments, sand grains, soil particles, and plant surfaces (4, 34, 58, 59). For turbid seawater, Yoon and Rosson (95) found that treatment with 10 μg of Tween 80 ml^{-1} and sonication with a half-wave step titanium horn (10 W for 30 s) yielded 10 times more attached bacteria than were obtained from untreated controls. Regarding marine sediments, Kuzhinovskii et al. (54) concluded that preliminary treatment by sonication was essential prior to AO staining, especially when working with finely dispersed silts. Dale (23) observed that homogenization of intertidal sediments (5 min at 23,000 rpm), however, consistently yielded larger cell numbers than did ultrasonification, grinding, or shaking by hand with the addition of glass beads. A complicated and time-consuming method for soils or sediments involving repeated homogenizations and density gradient centrifugations has been described (9).

Epiphytic or other surface-associated bacteria may be detached and dispersed by using a stomacher. Samples are placed in sterile bags with diluent water; the bags are then placed in a stomacher, where they are vigorously pounded by the machine's paddles. Stomaching yielded higher epiphytic bacterial counts than did homogenizing (34, 35), and a 5-min treatment in a Colworth Stomacher-400 (A.J. Seward Ltd., London, England) has been recommended (32). Baker (8) also reported that stomaching is the best method for removing epiphytic bacteria. For removal of bacteria from macrophyte-derived lignocellulose particles, it has been shown that sonication of samples for 90 s at a setting of 45 (Sonic Dismembranator model 300; Fisher Scientific) yielded the highest bacterial counts (11). For a variety of sample types, Velji and Albright (91) found that sonication (Biosonik II, 4-mm probe, 100 W for 30 s) after 15 to 30 min of pretreatment in $NaPP_i$ (0.1 or 0.01 M depending on sample type) was superior to vortex mixing with or without $NaPP_i$.

Bacteria have also been dislodged from sediment particles by ultrasonic treatment in sonifying ice-water baths. A sonification time of 2.5 min has been found to yield the optimum number of attached bacteria (30), although others recommend more gentle sonication for longer time intervals. For example, Schallenberg et al. (75) sonicated diluted sediment samples in a Branson ultrasonic cleaner for 30 min with 0.01 M $NaPP_i$, following the protocol of Duarte et al. (28). For some purposes (e.g., unpolluted water samples), vigorous hand shaking without the addition of dispersants has been considered sufficient (44), and in one case this was considered appropriate even for soil samples (14). However, in most cases, detachment, disaggregation, and uniform dispersion of bacteria by combinations of chemical and physical treatment are recommended (91).

Membrane Filters

Polycarbonate Nuclepore membrane filters have been most commonly used for direct counts. Anopore aluminum oxide membrane filters (pore size, 0.2 μm) yielded cell counts 21 to 33% higher than those of Nuclepore track-etched polycarbonate membrane filters, and the higher flow rates obtained with the Anopore filters also allowed lower vacuum pressures to be used (46). Despite these apparent advantages, Anopore filters have not been used frequently, perhaps because of their higher cost. Polycarbonate filters have been shown to be superior to cellulose-acetate filters, although an earlier filter comparison study recommended either cellulose-ester or polycarbonate membrane filters (45). Bowden (15) discovered that estuarine bacterial counts on 0.2-μm polycarbonate Nuclepore filters were significantly higher ($P < 0.001$) than counts on 0.2-μm Sartorius cellulose filters, whereas Pomeroy et al. (64) found no significant difference between counts on the same filter types. Counts on Nuclepore filters were not significantly different from those obtained by scanning electron microscopy in Bowden's (15) study.

Filters should be prestained black to reduce background fluorescence and provide the high contrast preferred for bacterial counts. Prestained filters may be purchased, although unstained filters may be easily stained in the laboratory prior to use. IB (Acid Black no. 107, 2 g $liter^{-1}$ in 2% acetic acid; Ciba-Geigy Corp.) is most frequently used to stain filters. We have successfully used Nuclepore filters stained with IB in petri dishes for 2 to 24 h. Filters stained in this way must be rinsed in filter-sterilized H_2O prior to use, and purchased black filters should also be wetted prior to sample addition.

The strength of applied vacuum pressure should be minimized to avoid disruption of fragile cells. Many investigators recommend filtration at a vacuum of less than 80 mm Hg (10.7 kPa) to avoid cell breakage, lysis, and penetration into the membrane (see, e.g., references 31 and 88), although others have used much higher vacuum without concern (e.g., 98 mm Hg [13.1 kPa] [2], 178 mm Hg [23.7 kPa] [65], and 608 mm Hg [81.1 kPa] [40]). We recommend even lower vacuum (<30 mm Hg [<4.0 kPa]) if microeukaryotes are also of interest, since vacuum at 75 mm Hg (10 kPa) causes losses of between 15 and 36% of nanoflagellates (13). Backing filters should be used to provide a more even distribution of vacuum (40). It is unfortunate that the vast majority of publications do not report the vacuum applied during sample filtration.

Filters with a nominal pore size of 0.2 μm are generally used, although many bacterial cells may actually pass through these filters. Polycarbonate membrane filters with a nominal pore size of 0.1 μm are now available, but their use for counting total bacteria in environmental samples has not been encountered in the literature. Using electron microscopy, Bae et al. (6) observed that small coccobacilli (<0.3 μm in diameter) made up 72% of natural soil bacterial populations and that many cells were less than 0.08 μm in diameter, too small to be seen by standard light or epifluorescent microscopic techniques. Likewise, oligotrophic marine systems may be dominated by small planktonic "ultramicrobacteria" less than 0.3 μm in diameter. These bacteria grow slowly but do not increase in size even when grown in nutrient-rich media (86).

TABLE 3. Final applied stain concentrations for the five sample types[a]

Stain	Final stain concn (μg/ml) (mean ± SD) in[b]:				
	Freshwater	Saltwater	Soil/sediment	Surfaces	Other
AO	78.0 ± 60.4 (*n* = 25)	145.6 ± 195.1 (*n* = 24)	138.6 ± 223.1 (*n* = 23)	57.5 ± 45.8 (*n* = 13)	154.4 ± 96.5 (*n* = 9)
DAPI	1.7 ± 2.8 (*n* = 15)	7.4 ± 13.9 (*n* = 12)	2.4 ± 2.5 (*n* = 7)	1.5 (*n* = 1)	1.0 (*n* = 1)

[a] See Table 1.

[b] *n* is the number of samples.

In reservoir water, bacterial cells with widths less than 0.18 μm contributed roughly 20% of total bacterial biomass, while the majority of cells were between 0.09 and 0.25 μm wide (71). A review of data on bacterial sizes in environmental samples is beyond the scope of this paper; however, it should be noted that significant numbers of cells in a variety of habitats are smaller than those traditionally accounted for by typical epifluorescent direct count methods.

Stain Concentration and Duration of Exposure

Jones and Simon (45) concluded that staining with 10 μg of AO ml^{-1}, with a contact time of 5 min, was the best method for staining aquatic bacteria. However, the majority of workers, both before and since 1975, have preferred to expose their samples to higher AO concentrations (e.g., 100 μg ml^{-1}) for short durations (Fig. 3). Considering all sample types, there is no general trend through the years in stain concentration and exposure time. However, higher concentrations of both DAPI and AO are recommended for samples containing sediments (33, 75, 91). Fry (33) reported using lower concentrations of both stains (AO at 5 μg ml^{-1} and DAPI at 0.05 μg ml^{-1}) for samples from German lakes but found that these concentrations were too low for samples from the Chesapeake Bay. There is some tendency to use higher AO and DAPI concentrations with saltwater and soil-sediment samples, although the variability in reported stain concentrations applied within these sample types is also high (Table 3).

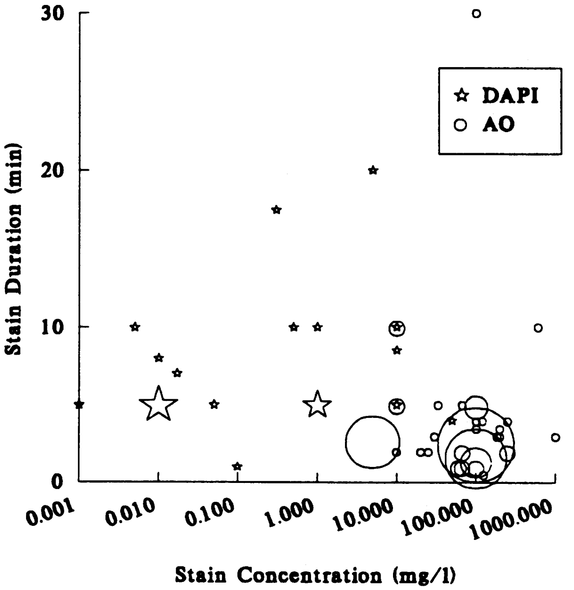

FIG. 3. Relationship between final AO or DAPI staining concentration and duration of staining for reviewed publications, where symbol size is proportional to the number of publications (range, 1 to 10).

Sample staining time is generally shorter for AO than for DAPI (Table 4), with the notable exception of one study examining tidal flat sediments (7). Longer exposure times are needed for soil-sediment samples, which tend to have more fluorochrome-binding detrital matter as well as mineral particles. The relationship between working-solution stain concentration (for either AO, DAPI, or both combined) and exposure time was quite weak (Fig. 3).

In addition to the effects of preservation and storage on bacterial biovolumes (see, e.g., reference 89), the type of fluorochrome used influences perceived cell sizes as well as numbers of bacteria observed. This must be considered when estimates of total bacterial biomass are desired. For example, Suzuki et al. (85) found that cell volumes obtained from measurement of DAPI-stained samples were on average 59% less than those estimated from measurement of AO-stained cells. If phototrophs are of interest, DAPI is recommended over AO, as AO may mask red chlorophyll autofluorescence (16).

The total volume of liquid sample filtered is also important, since it affects the eveness of distribution of bacteria on filter surfaces. Large errors can be associated with changes in the sample volume filtered (45). In addition to appropriate dispersion (see above), a minimum volume of 2 ml is recommended for the most commonly used 25-mm-diameter membrane filters, and volumes from 5 to 10 ml are preferred (32, 45). Samples are easily diluted with particle-free (0.2-μm-pore-size filtered) water prior to staining and filtration; however, applied AO or DAPI volumes should be adjusted accordingly to maintain an adequate stain concentration.

High variability in both stain concentration and exposure time (Tables 3 and 4), indicates the lack of a standard method for even the most common sample types. Stain concentrations vary by as much as 3 orders of magnitude even for the same sample type. Some researchers stain under ambient light conditions, while others recommend staining samples in the dark (see, e.g., references 46 and 70). Variations in staining techniques (e.g., filter-stain or stain-filter), filter types, stain types, concentrations and durations of exposure, and post-preparation counting methods may influence results. However, there is a prevailing impression that fluorescence microscopy

TABLE 4. Stain exposure times for the five sample types[a] when using either AO or DAPI

Stain	Exposure time (min) (mean ± SD)				
	Freshwater	Saltwater	Soil/sediment	Surfaces	Other
AO	3.1 ± 1.8	3.5 ± 3.8	4.5 ± 6.1	2.8 ± 2.5	2.3 ± 1.3
DAPI	8.4 ± 6.5	9.6 ± 6.2	10.0 ± 5.0	5.5	5.0

[a] See Table 1.

[b] Number of samples is the same as in Table 3.

has led to general agreement among researchers and that standard "methodological homogeneity" exists (see, e.g., reference 36). Currently, however, numerous methods (each yielding different counts for any given sample) are equally accepted.

Counting Methods

The same papers examined for information on sample preservatives were also reviewed for information on bacterial count strategies. Only 56% of these papers provided any information on how bacteria were counted following sample preparation. The magnification at which cells were enumerated was reported in only 35% of reviewed papers. The median and modal magnification used for direct counts of bacteria on membrane filters was ×1,250 (range, ×540 to ×1,875, $n = 53$).

Typically, numbers of bacteria are counted within squares of an ocular graticule or Whipple grid at ×1,250. Counts should be obtained from randomly located fields covering a wide area of the filter, although extreme edges of the filter should be avoided. Counting cells in fields located along two central transects positioned at right angles and not examining the filter while fields are being changed to ensure randomness have been recommended (32, 33). Traditionally, researchers have counted numbers of cells on upper surfaces of opaque particles and simply doubled this number, assuming that equal numbers of bacteria were on both sides of any large mineral or detrital particle. Turbid samples should be diluted so that the proportion of the field of view covered by particles does not exceed 40 to 70% (18, 37).

Recently, different means of accounting for the masking effects of sediment or detrital particles have been described in attempts to standardize counting procedures. Schallenberg et al. (75) have used a measure of turbidity (A_{750}) to indicate masking and have used the quartz-corrected H_2O content of various sediment types to develop a linear relationship between masking and the integrated (in situ × experimental) dilution of various sediments. This relationship makes it possible to correct for masking by sediment particles, thereby improving accuracy. Others attempt to remove most sediments following dispersion. For example, Kaplan and Bott (47) detach bacteria by adding glycerol followed by centrifugation to pellet sediment particles.

The literature was evenly split between studies in which a minimum number of cells (52%) or a minimum number of fields or grids (51%) were counted. Unfortunately, 17 of the 42 papers reporting studies in which a minimum number of fields were counted did not report the magnification used, making this information useless. Ocular grids are frequently used to delimit an area within the field of view in which cells will be counted; alternatively, all cells within the field of view are counted. Nine papers described studies in which a minimum number of both cells and fields or grids was counted, whereas four papers described studies in which either a minimum number of cells or a minimum number of fields was counted. Only eight papers described attempts to replicate counts by preparing more than one filter per sample. No relationship between count strategy and sample type existed.

The precision of counts depends on the number of bacteria counted. Assuming a Poisson distribution of bacteria upon membrane filters (33, 43, 44, 46), the 95% confidence intervals are approximately twice the square root of the number of bacteria counted, regardless of the number of fields or grids observed. To reduce the 95% confidence interval to ±10% of the mean (assuming a Poisson distribution), most researchers count at least 400 cells per filter. However, some investigators have found that fewer cells need be counted. Frequently, a minimum of 200 cells per filter are counted (Fig. 4). A study examining various levels of replication for sediment bacterial counts found that better results were obtained by counting five fields with roughly 30 cells per field on four replicate filters than counting all bacteria in 20 fields on a single filter (58). In this case and others (see, e.g., reference 51), replication at the level of subsamples and filters is recommended. It may be better to count fewer cells on two to four replicate filters than to count a greater number on a single filter (33; however, see reference 52).

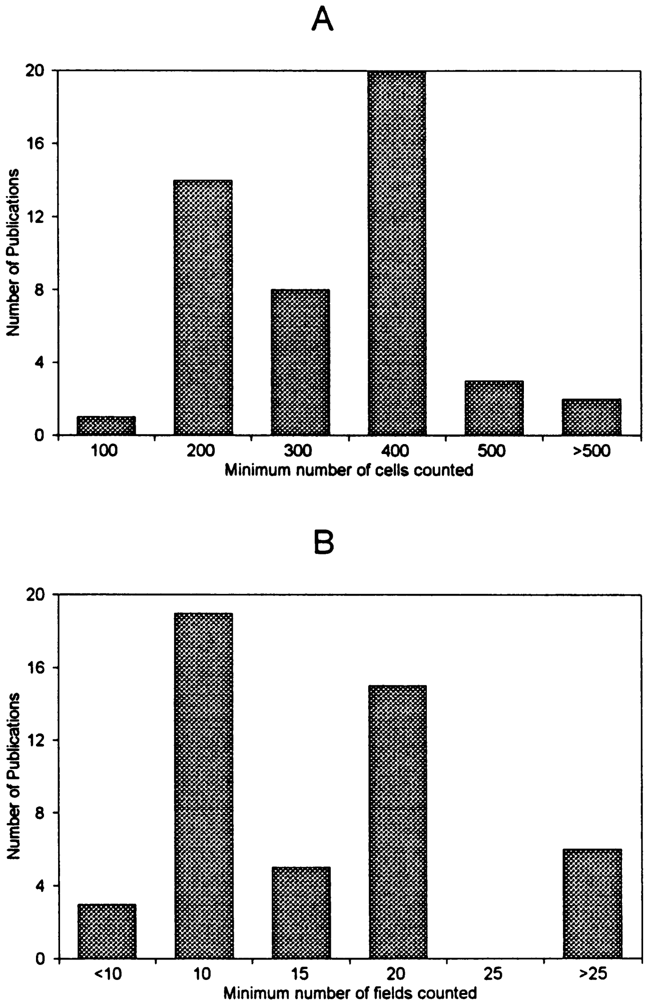

FIG. 4. Frequency distributions for numbers of reviewed papers reporting a minimum number of bacterial cells counted (A) or microscope fields viewed (B) per filter.

Lebedeva and Shumakova (55) observed empirical distributions which displayed statistically significant differences from the theoretical Poisson distribution. These authors prepared a nomogram making it possible to determine the number of fields which must be counted at a given cell concentration to obtain a given degree of accuracy. With their effective dispersion method (see above), Velji and Albright (92) obtained samples yielding stable coefficients of variation (CVs) after counting 10 grids, compared with untreated samples for which CVs did not stabilize even when up to 30 grids were counted.

The majority of papers reviewed described experiments in which bacteria were enumerated in a minimum of either 10 or 20 fields or grids. Regardless of count strategy, for counts based on some minimum number of fields or grids, it is best to dilute samples to a point yielding fewer than 50 cells per field or grid. It has been stated that counting 25 to 30 bacteria per field is satisfactory with DAPI, for which photofading is not so much of a problem (33). AO-stained samples may fade more rapidly, and cells within a grid may become invisible before counts can be completed at high densities. A potential solution suggested by some researchers is the use of slide mountants such as Citifluor AF2, which retard photofading of stained bacteria (94), or addition of cationic brighteners directly to staining solutions (e.g., Uvitex AN as used in reference 62).

Background counts of sample blanks should be carried out to ensure that all solutions and apparatus are free of bacteria. Mean cell densities obtained from blanks (filters prepared at the beginning and end of each staining session with all reagents but without sample addition) should be subtracted from the final calculated densities. Blank densities should be less than 5% of total cell densities; in the absence of contamination, they are normally less than this.

For a complete consideration of the impacts of counting and sampling strategies, the works of Kirchman et al. cannot be ignored (51, 52). Examining count variation at several levels of replication, these investigators found that variation among microscopic fields was highest and contributed from 62 to 80% of total variance (51). On the basis of cost and error analysis, Kirchman (52) suggests that two preserved subsamples be examined, that one filter per subsample be prepared, and that more than 10 microscope fields (containing approximately 30 cells per field) be examined on each of the filters.

Investigator Bias

A thorny issue, rarely discussed, is the bias introduced by different observers at the microscope. Differences in estimates between observers are likely to depend on individual interpretations of what actually constitutes a countable bacterial cell. Because of differences in which particular objects are recognized as stained bacterial cells, between-operator variation may be large (see reference 52 and references therein).

To partially address the problem of subjectivity involved in identifying fluorescing objects as bacterial cells, we evaluated count data obtained by three different observers working in our laboratory (Fig. 5). Each individual examined the same 10 prepared slides following the application of our DAPI staining protocol (see below). Samples were of whole water obtained from Sayers Lake, Centre County, Pa., during September and October 1989.

Bacterial densities obtained by the three observers were not significantly different (one-way analysis of variance; $P = 0.18$). Count data conformed to normality and equality of variance assumptions, with the CV over all samples being 30.0, 21.7, and 23.9% for investigators 1 through 3, respectively. The average CV between counts obtained by each person was 16.9% (range, 7.8 to 34.4%; $n = 10$). This is comparable to mean CVs obtained in previous work (51) at the levels of microscope fields (16.8%; $n = 75$), and individual filters (10.0%; $n = 15$). General trends in estimated bacterial densities were consistent between observers. For example, samples 2 to 5 and 10 had the lowest bacterial counts for all three investigators in our laboratory (Fig. 5).

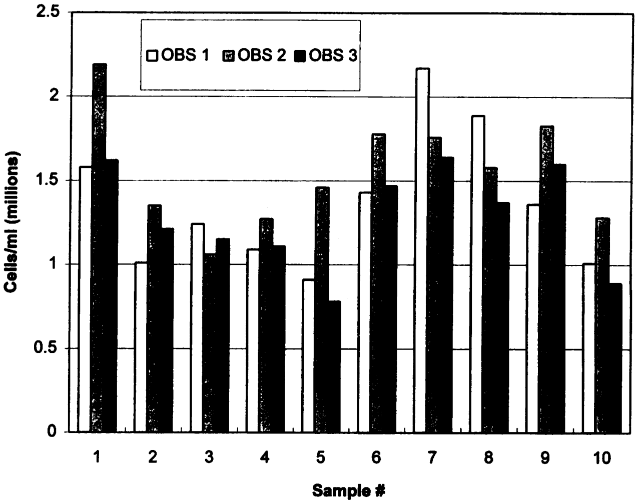

FIG. 5. Total bacterial densities obtained by three individual observers examining the same prepared (DAPI-stained and membrane-filtered) lake water samples.

The problem of investigator bias remains, however, since variation in counts may be partially attributable to patchy cell distributions on filter surfaces; i.e., not all fields of view contain the same number of cells, and the same fields on each filter are not viewed by each person. Individual bias does not appear to constitute an unusually large source of variation in count results relative to other sources of variation within the methodology. At best, we can echo the call of others (see, e.g., references 52 and 60) to recognize the subjectivity inherent in any direct count method. Observers must be appropriately trained by those with greater experience in the recognition of bacterial morphologies.

PROBLEMS WITH THE METHODS

Count differences based on choice of stain are addressed by several papers in which bacterial stain comparisons involving DAPI and/or AO have been made (Table 5). Meanwhile, other potentially significant differences between methods remain largely overlooked. Schallenberg et al. (75) address problems associated with inefficient DAPI staining and particle-masking effects encountered when working with sediment samples. Kepner and Pratt (50) have shown that DAPI counts underestimate those obtained with AO in the presence of fine sediments. Similarly, Suzuki et al. (85) have found that DAPI counts average only 70% of AO counts in coastal seawater samples. Previous work had already indicated that AO may

TABLE 5. Papers in which DAPI and/or AO is compared with other fluorochrome stains for estimates of total bacterial abundances

Reference	Tested stains
25	Euchrysine-2GNX and AO
45	Euchrysine-2GNX and AO
65	DAPI and AO
70	DAPI and AO
63	DAPI, AO, and bisbenzimide dyes (Hoechst 33258 and 33342)
64	DAPI and AO
81	DAPI, AO, and Hoechst 33258
12	AO, acriflavine, and Hoescht 33258
60	DAPI and AO
54	Erythrosine, fluorescamine, and AO
50	DAPI and AO
85	DAPI and AO

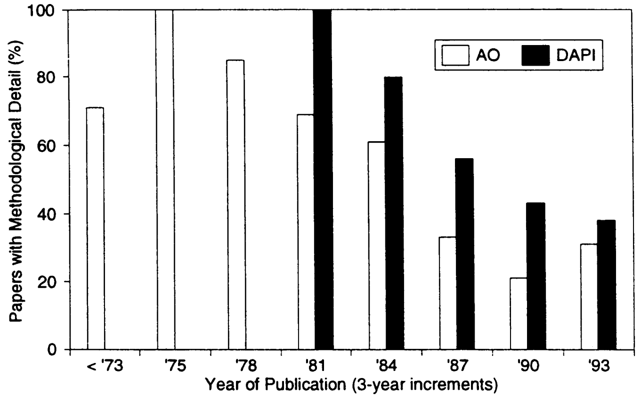

FIG. 6. Percentage of reviewed publications containing "methodological detail" (i.e., papers containing any information on applied stain concentrations and exposure times), over time.

yield higher counts than DAPI in seawater samples (see reference 60 and references therein). Apparently, some AO-stained cells are not visibly stained with DAPI, although the mechanisms responsible for this and resulting count differences in certain types of environmental samples remain unknown.

An epifluorescent direct count method for total bacterial enumeration with AO was approved by the Standard Methods Committee of the American Public Health Association (2), and a similar protocol was approved as an American Society for Testing and Materials Standard Test Method (3; reapproved in 1990). Nevertheless, we find the lack of agreement on standard procedures to be particularly problematic given the recent trend for dismissing practical methodological information in scientific publications. In this review, we considered papers including any information on applied stain concentrations and exposure times to be "methodologically detailed." The percentage of papers including such detail has been dropping steadily in recent years (Fig. 6), first for papers describing experiments with AO and now for those describing the use of DAPI. Papers providing information on preservatives, dispersion techniques, filter types, and counting strategies are equally rare. If current trends continue, researchers might begin to see the following. . . "Bacteria were enumerated by microscopic direct counts," as a succinct yet sufficiently "detailed" description of the method used. Certainly, if this point is reached, our ability to make interstudy comparisons will be completely eroded.

As a starting point directed toward unifying approaches to performing epifluorescent bacterial direct counts, we present the following generalized procedure. This protocol is a synthesis of reviewed methods and is compatible with several previously recommended methods (see, e.g., references 2, 3, 33, 77, and 88). The simplicity of the following procedure makes it attractive for the routine estimation of bacterial densities in a variety of environmental sample types.

A GENERALIZED PROCEDURE

Reagents

(i) Fixative: 10% (wt/vol) P_i-buffered GTA
0.40 g of NaH_2PO_4
1.23 g of Na_2HPO_4
80 ml of distilled H_2O
20 ml of 50% (wt/wt) GTA

(ii) Dispersant: 0.1 M tetrasodium PP_i
44.61 g of $Na_4P_2O_7 \cdot 10H_2O$
1.0 liters of distilled H_2O

(iii) Fluorochrome stains: stock solutions
100 μg of DAPI ml^{-1}
10 mg of DAPI
95 ml of distilled H_2O
5 ml of 50% (wt/wt) GTA

or

1,000 μg of AO ml^{-1}
100 mg of AO
95 ml of distilled H_2O
5 ml of 50% (wt/wt) GTA

Store stock solutions in the dark at 4°C

(iv) IB solution:
0.2 g of IB
95 ml of 2% acetic acid
5 ml of 50% (wt/wt) GTA

(v) Diluent and rinse water:
500 ml of distilled H_2O (filter sterilized [pore size, 0.2 μm] and autoclaved)
25 ml of 50% (wt/wt) GTA

Make fresh daily

(vi) Immersion oil: Non-fluorescing, non-drying immersion oil (type FF; Cargille Laboratories Inc.)

(vii) Reagent preservative: 50% (wt/wt) GTA

Diluent water and stains should be preserved with glutaraldehyde (final concentration, 2.5%). Reagents other than immersion oil should be stored refrigerated (4°C) in the dark and filter sterilized (pore size 0.2 μm) just prior to use. All reagents must be sterile, particle free, and at room temperature (21°C) prior to use.

Apparatus

(i) Epifluorescence microscope: UV illuminator (e.g., 200-, 100-, or 50-W high-pressure mercury lamp) and flat-field 100× oil immersion, fluorescence-free objective with high numerical aperture. Fluorescence intensity increases exponentially with the increase in numerical aperture. A minimum total magnification of ×1,000 is required, although ×1,250 or higher is preferred.

(ii) Light filters: Combination excitation and barrier filters; for AO, blue excitation (450- to 490-nm wide-bandpass filter); for DAPI, UV excitation (narrow-band 365/366 nm). Available from K. Zeiss, Lietz, Olympus, Nikon, or equivalent (see manufacturer recommendations).

(iii) Ocular graticule: Stage-micrometer-calibrated 10 by 10 eye-piece reticule, Whipple grid, or equivalent.

(iv) Membrane filters: 25-mm-diameter, 0.2-μm-pore-size, prestained black polycarbonate membrane filters (Nuclepore Corp., Poretics Corp., or equivalent).

(v) Backing filters: 25-mm-diameter, 1.0- to 8.0-μm-pore-size cellulose-acetate or other cellulosic filters (Millipore Corp., Poretics, Corp., or equivalent).

(vi) Filter tower apparatus: 25-mm filter supports and funnels, a regulated vacuum pump (a vacuum manifold for processing multiple samples at one time is optional).

(vii) Pipettes: 0.1-, 1.0-, and 5.0-ml pipettes and sterile pipette tips (adjustable autopipettes preferred); micropipettes and sterile tips for dispensing volumes from 10 to 100 μl.

(viii) Syringes: 5-ml, sterile disposable syringes and autoclaved (or disposable) syringe filter holders and filters (0.2 μm).

(ix) Vortex mixer and one of the following mechanical dispersant devices: ultrasound bath (e.g., Bransonic), stomacher (e.g., Colworth Stomacher-400 [A. J. Seward Ltd., London, England]), Waring blender or other homogenizer (e.g., Ultra-Turrax), probe-type sonicator (e.g., BioSonik II, Ultratip Labsonic System [Lab-Line Instruments, Inc.], Sonic Dismembranator model 300 [Fisher Scientific]).

(x) Miscellaneous: Clean microslides, coverglasses (no. 1), filter forceps, sterile glass bottles for solution storage (use dark glass or wrap bottles with aluminum foil to protect light-sensitive stains), slide trays for storage of prepared slides, hand-held or bar counter.

Procedure

(i) Collect samples in sterile containers and preserve with buffered GTA (final concentration, 1%). For example, add 9 ml of water or sediment slurry sample to a sterile 15-ml screw-top sample tube already containing 1 ml of ice-cold fixative. Fixed samples may be stored refrigerated (4°C in the dark) for up to 3 weeks, but it is best to prepare slides within a few days of collection, because decreases in numbers have been observed even when samples are preserved in aldehyde solutions under refrigeration (89).

(ii) If prestained membrane filters are unavailable, regular filters must be stained for 2 to 24 h in IB solution in a sterile petri dish. Rinse the IB-stained filters twice in sterile, particle-free distilled H_2O prior to use.

(iii) Prior to staining, allow samples and other reagents to reach room temperature. Place backing filter on filter tower apparatus support, prewetted with one drop of sterile diluent H_2O. Top with prewetted black membrane filter (pore size, 0.2 μm), keeping the shiny side of the filter face-up. Replace filter tower top (funnel).

(iv) Disperse bacteria and disrupt bacterial aggregates by adding PP_i (final concentration, 0.01 M) and treating the sample with the selected dispersant device (sonifying bath, stomacher, etc. [see above]). Sediment and soil samples will require predilution in sterile particle-free H_2O (up to 1:1,000 [wt/vol], depending on the initial H_2O content of the material). Care must be taken to avoid overheating samples or disrupting cells with overly harsh treatment during homogenization.

(v) Sample volume for staining must be adjusted on the basis of bacterial density, and this must be determined by a process of trial and error. Sample volumes ranging from 0.05 to 5.00 ml are appropriate for most natural surface water samples. Use DAPI at a final staining concentration of 0.1 $\mu g\ ml^{-1}$ for relatively clean aquatic samples and 0.5 $\mu g\ ml^{-1}$ for sediment-bearing samples, epiphyte suspensions, or other samples containing large proportions of nonbacterial particulate material. A final concentration of 100 $\mu g\ ml^{-1}$ is normally sufficient for all sample types when using AO. If bacterial fluorescence is too faint, fluorochrome concentrations may be increased. Likewise, if background fluorescence is overwhelming, concentrations may be decreased. Information summarized in this review may serve as a guide to appropriate stain concentrations for a given sample type.

(vi) Stain samples in the filter tower to avoid an additional source of sample contamination. Combine diluent H_2O, sample, and fluorochrome (DAPI or AO) (in that order) to obtain a total liquid volume not less than 2.0 ml. For example, to stain 0.50 ml of lake water with DAPI, add 1.48 ml of diluent H_2O, 0.50 ml of pretreated sample, and 20 μl of 10-$\mu g \cdot ml^{-1}$ DAPI.

(vii) Perform staining in a darkened room. Immediately following stain addition, swirl funnel contents to promote thorough mixing of stain and sample. Allow AO to react with sample for 3 min (DAPI for 7 min), while occasionally swirling filter funnel contents.

(viii) After the allotted staining time, draw funnel contents through the filter tower under low vacuum (<30 mm Hg [<4.0 kPa]). If contents take more than 1 or 2 min to pass the filter, samples will require further dilution. Rinsing the membrane filter with a volume of water approximately equal to that of the sample removes excess stain, enhancing image contrast (32). Just before all sample passes the filter, rinse around the base of the filter funnel with two separate aliquots (1.0 ml each) of diluent H_2O. Continue vacuum until all liquid is gone.

(ix) Release vacuum, disassemble filter tower apparatus, and remove membrane filter with forceps. The backing filter may remain in place and be reused.

(x) Place a small drop of immersion oil on an appropriately labeled, acetone-cleaned glass microslide. Make sure that the filter stays sample side up. Place another drop of immersion oil on top of the black membrane filter, and top with a clean coverglass. Allow the oil to spread, and gently press out air bubbles from beneath the coverglass if necessary.

Counting and Calculations

(i) Despite reports of successful storage under refrigeration (see, e.g., reference 65) for periods of up to 1 month, we recommend that slides be counted as soon as possible following preparation, ideally on the same day. If slides must be stored, refrigerate (4°C) and minimize exposure to light.

(ii) Determine the effective filtration area of the apparatus being used. Only a portion of the 25-mm-diameter filter area is actually available for the deposition of cells, as the inner diameter of the filter funnel is always less than 25 mm.

(iii) Determine the area of the field of the ocular (e.g., Whipple) grid, or eyepiece graticule being used, at the magnification to be used for counting by using a stage-micrometer.

(iv) Count cells falling within the grid in randomly located fields. To avoid bias, the sample should not be observed while fields are being changed. We recommend counting a minimum of 400 cells per filter falling within a minimum of 20 fields of view. Assuming a Poisson distribution of cells on the filter, this should yield an acceptable 95% confidence interval of roughly ±10% of the mean (44). When AO is used, all cells should be counted regardless of color (orange, reddish, or green).

(v) A hand-held or bar counter, of the type commonly used for enumerating blood cells, is useful for keeping track of both numbers of grids and numbers of bacteria observed. Bacteria are most easily counted in a darkened room.

(vi) Calculate bacterial density in the original sample by using the formula

$$\text{Bacteria (cells per milliliter)} = (N \times A_t)/(d \times V_f \times G \times A_g)$$

where N is the number of cells counted, A_t is the effective area of the filter (square millimeters or square micrometers), A_g is the area of the counting grid (square millimeters or square micrometers), V_f is the volume of diluted sample filtered (milliliters), and d is the dilution factor ($V_{\text{final}}/V_{\text{sample}}$) (account for addition of preservative and dispersant as well as any dilution prior to addition of sample to the filter funnel).

We recommend the preparation and counting of two filters per sample (however, see reference 52). Bacterial densities obtained from appropriate blanks should be subtracted from the final calculated densities. Typically, sediment or soil sample cell densities are converted to numbers of cells per sediment volume (e.g., cubic centimeters) or numbers per soil mass (e.g., grams [dry weight]). Surface-associated (e.g., epilithic or epiphytic) bacterial densities are usually expressed as numbers per unit area (e.g., square millimeters).

Recommended Reporting

We recommend that, at a minimum, the following information be included when reporting bacterial densities.

(i) Preservative:	Type and final concentration, conditions, and duration of sample and prepared slide storage.
(ii) Stain:	Type, final concentration, and duration of staining.
(iii) Filters:	Type and nominal pore size.
(iv) Counting:	Count strategy (minimum number of cells and/or grids), total magnification used.

Supplemental information which would also be useful might include the following.

(i) Dispersant:	Type and final concentration, dispersion treatment (particularly for surface-associated bacteria).
(ii) Microscope:	Nominal wavelengths of filter sets.
(iii) Vacuum:	Strength of vacuum applied during filtration.

Description of the method should be sufficiently detailed to allow repeatability as well as an assessment of comparability between studies. As more factors which significantly affect bacterial count results are discovered, it is by necessity that these be considered in our efforts to reliably estimate bacterial densities in the real world.

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